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International application number: PCT/US05/006638

International filing date: 28 February 2005 (28.02.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/549,306
Filing date: 02 March 2004 (02.03.2004)

Date of receipt at the International Bureau: 15 July 2005 (15.07.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

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RELATED PCT APPLICATION NUMBER: *PCT/US05/06638*

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22151 U.S. PTO 60/549306 030204

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Robert J.	MAIER	Athens, GA

☐ *Additional inventors are being named on the____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 53
☐ CD(s), Number
☐ Drawing(s) *Number of Sheets*
☐ Other (specify)
☐ Application Data Sheet. See 37 CFR 1.76

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
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Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME **Bradford G. Addison**

TELEPHONE **317-231-7253**

Date **3/2/2004**

REGISTRATION NO. *(if appropriate)* **41,486**

Docket Number: **31725-74647**

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11 South Meridian Street
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(317) 236-1313

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group:	Unknown
Attorney Docket:	31725-74647
Applicant:	Robert J. Maier
Invention:	**Bacterial Strains**
Serial No:	Unknown
Filed:	March 2, 2004

CERTIFICATE UNDER 37 C.F.R. § 1.10

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Date of deposit: March 2, 2004

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Garla L. Twyman
Signature

Garla L. Twyman
Typed or Printed Name

Enclosure
Indianapolis, Indiana
(317) 231-7388

Statement of Future Use of Salmonella strains R. J. Maier Feb 10, 2004

The attached data demonstrates that use of the strains unable to use molecular hydrogen as a pre-challenge to a lethal dose of the virulent (H_2-oxidizing) strain results in protection of the animal from disease (salmonella-induced typhoid fever). Not a single animal died or showed disease symptoms if it was first inoculated with the mutant strain. It would be expected that use of this salmonella strain (triple mutant) as a vaccine, or the creation of similar strains of Yersinia, Shigella, E. coli, or Campylobacter, or even other salmonella species would also give such a protective affect against shigellosis, yersinial infections (enterocolitis or bubonic plague), campylobacteriosis, or a wide range of *E. coli* infections of the urinary tract. These would apply to human infections as well as to livestock. Probably the most likely use of these stains would be to prevent diarrheal illnesses and typhoid fever. It is likely that the virulent strains never pass the mesenteric lymph nodes to overwhelm the host, if the host is first challenged with the strain unable to use H_2.

20 Balb/c female mice (born on Sept. 1, 2003). Mice were obtained from:

The Jackson Laboratory
610 Main Street
Bar Harbor, Maine 04609-1500

Oct. 14, 2003 mice were inoculated with 10^6 cells of *Salmonella typhimurium* , triple mutant. This mutant had the following deletions:

1. (STM 3150, STM 3149, STM 3148, STM 3147)
2. (STM 1539, STM 1538)
3. (STM 1786, STM 1787)

All of the mice survived.

On Dec. 4, 2003 the 20 mice were inoculated with:

100 µl containing 5 x 10^6 cells/ 100 µl of wild type* were administered to the mice by oral gavage. Mice were checked daily, and all were fine.

On Dec. 15, every mouse was fine.

Dec. 16 every mouse was fine; at this time all were euthanized.

Further characterization of this phenomena (using the H_2 oxidizing strain as a challenge/vaccine) will include determining the dose dependency of the protection affect.

*This wild type strain is lethal. Previous experiments have shown that by day 11 all mice inoculated with this wild type strain at 1 x 10^6 cell dose per animal were dead.

Principal Investigator/Program Director (Last, first, middle): Maier, Robert J.

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Salmonella are considered a category B priority biodefense concern, mainly due to their easy introduction and subsequent transmission through food or liquid. Both *Salmonella enterica* serovars **typhimurium** and **typhi** contain genes for enzymes that are predicted to use molecular hydrogen (H_2) as their substrate (i.e. energy source), but the role of these enzymes for maintenance of and pathogenicity by *salmonellas* within animal hosts has never been addressed. Whole genome sequence analysis reveals that three separate membrane-associated nickel-containing H_2 utilizing enzymes may function in *Salmonella enterica* typhimurium, and our recent enzyme assays on H_2 utilization of a virulent strain indicate substantial H_2 utilizing activity that is coupled to a cytochrome-dependent respiratory chain. Therefore, it is likely that *Salmonella* gleans energy for growth by using H_2 produced from within the host animal, like the type of host hydrogen-produced growth we recently demonstrated for the human pathogen *Helicobacter pylori*. This possibility will be tested by generating H_2 uptake negative bacterial mutants and comparing their virulence with the parent strain by use of a mouse model. When the virulence studies are done, the mutants will be used to associate the H_2-binding affinity of *S. typhimurium* with particular hydrogenases that are important for virulence in the host. The results are expected to be readily applicable to human disease caused by *Salmonella*, as H_2 is an available substrate in the human body, and one not used by the human host. The unique properties of hydrogenases as nickel-containing enzymes makes them attractive targets for eradication of pathogenic bacteria that use H_2 in animal hosts.

PERFORMANCE SITE(S) *(organization, city, state)*

Department of Microbiology, University of Georgia, Athens, Georgia

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Robert J. Maier	Dept. of Microbiology, Univ. of Georgia	PI
Adriana Olczak	Dept. of Microbiology, Univ. of Georgia	Laboratory Technician II (M.S. Degree)
Graduate Student (to be named)	Dept. of Microbiology, Univ. of Georgia	Research Assistant

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

A. Specific Aims

The primary goal is to address the role of H_2, present in animals as a consequence of the fermentative metabolism by the normal colonic flora, as a substrate for maintenance/virulence of pathogenic *Salmonella*. *Salmonella* has the capacity to use H_2 with O_2 as the terminal acceptor (our preliminary results), presumably via several H_2-utilizing enzymes (three membrane-associated NiFe hydrogenases based on the whole genome sequence annotation). Importantly, the substrate (H_2) is known to be present within animal tissue. This is based on our recently published work on microelectrode H_2 measurements within the stomachs of live mice, and our similar unpublished work on H_2 levels within other (mouse) tissues. The aims can be divided into two categories, one based on the use of an animal model to study virulence associated with H_2 use, and the other on biochemical/physiological properties associated with the role of H_2 oxidation. They are:

1) **Determine the roles of H_2 utilization in virulence.** Our primary aim is to address the role of H_2 utilization by *Salmonella typhimurium* (Salmonella enterica serovar typhimurium) in causing disease in animals. This will be approached by systematic targeted mutagenesis combined with testing virulence in a mouse model. Four mutants will initially be generated. Three of these will be single mutants, one in each of the 3 uptake hydrogenases (predicted from the genome sequence). A triple mutant will also be made that lacks all H_2 using ability. The mutant strains will be generated in the lab of a collaborator listed herein (Prof. John Gunn) who is an expert on *S. typhimurium* mutagenesis and the bacterium's physiology. Part of the proposed budget is for Dr. Gunns portion of the proposed work.

2) **Study biochemical/physiological properties of the hydrogenases to bolster the conclusions from aim #1.** By use of the mutant strains from aim #1 as well as from studying other mutants lacking two of the three putative hydrogenases we will determine the roles of each H_2 utilizing enzyme in reducing a cytochrome-dependent respiratory chain that culminates in oxygen-dependent respiration. This will be especially important after we know which hydrogenases are the most important for virulence. Also, by use of the mutants we will address the binding affinity of the important hydrogenases for the substrate (molecular hydrogen) in whole cells. This will correlate the predicted catalytic turnover for H_2 activation with actual substrate levels we have measured in the animal.

It seems most logical to first identify the hydrogenases (if any) that are important to virulence of the bacterium (aim #1), and then to focus on the properties (like energy-generating characteristics) of that one (or more) enzyme(s).

B. Background and Significance

Salmonella Physiology and Pathogenesis

Salmonella strains can cause disease ranging from localized gastroenteritis to systemic infections leading to septic shock and death (1). *S. typhimurium* is one of the most common causes of diarrhea and is transmitted primarily by ingestion of contaminated food. Although the infected individual recovers from the abdominal pain and diarrhea, this individual continues to shed bacteria for up to 3 months and some individuals for up to a year (1). Therefore it is clear the bacteria have the capability of surviving and growing in the host. Although many cases of salmonellosis are linked to raw egg products and poultry, documented cases of disease have been transmitted via milk, handling pet reptiles, or even from marijuana (1). *S. typhi* is a more serious pathogen, causing

typhoid fever. It causes many deaths worldwide. Some individuals become colonized by the bacteria and suffer no symptoms, but they are a major reservoir to enable the spread of the bacterium. The *S. typhi* multiply in the liver and spleen (tissues in which we have measured H_2, see Preliminary Results) for up to a month before being released into the bloodstream, causing a high fever and anorexia. After an infection subsides, the bacteria can occupy the gall bladder, where they maintain a reservoir for spreading the disease to other individuals. When individuals carry *S. typhi* in their gallbladder, the bacteria can be shed in the feces for many years, and introduced into food, resulting in dissemination. According to the genome sequence analysis, both *S. typhimurium* and *S. typhi* have genes for a number of membrane bound H_2 oxidizing hydrogenases enzymes (2). These have been studied very little, and most importantly the roles of these H_2 oxidizing enzymes in providing maintenance energy for the bacteria to reside in animal hosts has not been addressed. Based on our recent results published in the November 29, 2002 SCIENCE, H_2 is an available substrate within animals, and the capacity for a widespread pathogen (the gastric pathogen *H. pylori*) to use it in the stomach is very important in the bacterium's persistence/virulence (3). Now we have measured molecular hydrogen levels in other tissues, including the spleen and the small intestine; hydrogen levels there are also considerably greater than the K_m for H_2 of (studied) membrane bound hydrogenases within H_2-oxidizing bacteria.

Virulence factors for *S. typhimurium* include Type III secretion system factors, a transporter of Mg^{++}, superoxide dismutase, adhesions, and Spv proteins (toxins important at many stages of virulence, one of which ADP-ribosylates actin) (1,4). When infected with *S. typhimurium*, mice develop a fatal systemic form of infection that resembles human typhoid fever rather than gastroenteritis (4,5). This could be taken as a flaw in using the mouse as an animal system to make conclusions about virulence of *S. typhimurium* per se (1), but has still been a useful model to make conclusions about virulence determinants likely to operate in both *Salmonella enterica* serovars typhi and typhimurium (5,6). Full virulence of *S. typhimurium* in a mouse model requires Mn(II) and Fe(II) transport systems, too (5), and resistance to antimicrobial peptides is important for survival in the host (7).

Hydrogenases in S. typhimurium

In the mid-1980's, *S. typhimurium* strain LT2 was shown to contain two immunologically distinct membrane-bound hydrogenase enzymes (8,9). This finding lead to the examination of other *S. typhimurium* isolates, and 3 hydrogenase enzymes (each with a homologue in *E. coli*) were studied in *S. typhimurium*. One of the hydrogenases was correlated with hydrogen-lyase dependent hydrogen evolution, whereas the two membrane bound enzymes were postulated to play roles in (anaerobic) H_2 uptake (8). Based on studies of natural isolates lacking one or more of the hydrogenases, it was proposed that at least one of the membrane bound hydrogenases (hydrogenase enzyme #2) functioned in anaerobic respiration-driven growth with nitrate as the terminal acceptor (9). Even with nitrate as the terminal acceptor it was believed that H_2 oxidation was linked to energy conservation (proton translocation events) via an anaerobic respiration pathway (9,10). As enlightening and pioneering as these studies were, none of the hydrogenase assays were done with O_2 as the terminal acceptor to address the possibility that one or more of these hydrogenases functioned to respire O_2 with H_2 as the reductant. It was presumed from earlier studies in *E. coli* that all reactions involving H_2 metabolism in enteric bacteria were related to anaerobic metabolism (11). However, now we know (from the complete genome sequence analysis) that *S. typhimurium* has the ability to carry out O_2-dependent respiration using several efficient O_2 reducing terminal oxidases (see 2). In addition, we have observed that all of the components for efficient cytochrome and quinone dependent proton gradient formation with electrons from H_2 are evident from the genome sequence of both of the *Salmonella enterica* serovars of interest here. It is difficult to

predict which of the 3 hydrogenases may be coupled to ATP generating respiration; for example all 3 have genes within their operons that encode membrane bound heme-b containing proteins, and cytochrome b are the electron acceptors for electrons from studied respiratory hydrogenases. Thus they all could be involved in energy generation. It would be expected that coupling of H_2 oxidation to O_2 reduction would be more favorable energetically (and more ATP gleaned) than if H_2 oxidation is strictly anaerobic.

At least two of the *S. typhimurium* hydrogenases are homologues of (studied) *E. coli* NiFe hydrogenases. In *E. coli* the two Ni-containing hydrogenases of most relevance to the proposal here are membrane-associated and they differ in the redox potentials at which they function to oxidize H_2 (35,36). Still, they both carry out H_2 oxidation and it was hypothesized (but not studied) that they may even be able to do so in a respiratory fashion with O_2 as the terminal acceptor (35). Another (the third) *E. coli* hydrogenase (36) has been studied very little and no role for it has been proposed (13). A fourth *E. coli* hydrogenase (also with some homology to one of the putative *Salmonella* hydrogenases) is proposed to function in a proton-translocating respiratory pathway to formate, essentially forming a formate hydrogenlyase complex (37). This complex uses externally-supplied formate and also contains nickel, and is proposed to be coupled to energy generation (albeit anaerobically) as well. Therefore, an *Salmonella typhimurium* hydrogenase that we may find that is not O_2 respiration-linked may still be very important in maintenance of the pathogen (via H_2 use) in animals, depending on the availability of electron acceptor sources. Indeed a range of organic terminal electron acceptors could be important in receiving electrons originating from H_2, but in the very low O_2 or possibly anaerobic environment areas in the animal host. For this reason, we need to initially determine the virulence role of the hydrogenases individually, and then address their (energy-generating) roles and their associated respiratory pathways.

When we found that the gastric pathogen *H. pylori* used H_2 as an important respiratory substrate to enable colonization of the stomachs of mice, we decided to re-investigate the role this small energetic substrate could play in virulence of another pathogen (*S. typhimurium*, see Preliminary Results). Most of the H_2 oxidation activity we observe is O_2-dependent and is inhibited by cyanide. Uptake type hydrogenases have unique nickel-containing active centers (12,13), so if H_2 use is a major factor for maintenance of the pathogen in animals, interfering with nickel uptake could render the pathogen energy starved.

Availability of Hydrogen in Animals

Recently, we showed that an H_2 uptake type hydrogenase (14) is an important bacterial characteristic to confer colonizing ability to *H. pylori* (3). Hydrogen oxidation is carried out by many diverse respiratory bacteria, as it is one of several possible reducing substrates common in nature. The low potential electrons can be coupled to energy conservation, and this ability helps *H. pylori* persist in the energy-poor environment of the gastric mucosa. H_2 has been measured as an excreted product from the intestinal tract of humans and rodents (33); this is due to its production by intestinal flora, and the gas has been speculated to be carried through the bloodstream (15,16). The source of this H_2 is from reactions associated with acetate or butyrate production by fermentative bacteria in the large intestine of animals (see 15). In addition to the stomach H_2 measurements we conducted previously (3), we have measured H_2 levels averaging over 50 μM in the livers of live mice. Hydrogen detected on the breath of humans is associated with the degree of digestibility of the complex carbohydrates that are ingested, with the more non-digestible (and therefore left for fermentative reactions in the colon) ones leading to higher H_2 levels (see 17 for one example).

The levels of H_2 we detected in mice far exceeded the affinity of the bacteria for the substrate, molecular hydrogen (3). This affinity was due to hydrogenase (based on lack of H_2 binding by a hydrogenase negative *H. pylori* mutant strain), so we concluded the H_2 utilizing

enzyme in the parent strain was usually saturated with H_2 in the host tissue. H_2 utilization is unique to bacteria, so that inhibitors of this enzyme may have applicability in reducing survival of the pathogen but with the advantage of not affecting the host. Also the unique (nickel-containing) active site of H_2 binding by NiFe-hydrogenases makes this enzyme a good target for the design of new and specific antibiotics.

C. Preliminary Results

Availability of molecular hydrogen in animals

By using a 50 μmeter microelectrode probe in conjunction with signal amplifiers we measured the levels of molecular hydrogen at the mucosal surface inside the stomachs of live (anesthetized) mice (3). From 31 measurements in 4 mice, we determined the average hydrogen concentration to be over 43μM (3). We have extended these measurements to include other tissues, ones of relevance to *Salmonella typhi* and *S. typhimurium* occupancy. The tables show the levels of hydrogen we measured in the liver of three mice (table 1), as well as in the spleen and the inside of the small intestine of a single mouse (table 2). Details of the methods can be found in ref 3 and 40.

Table 1. Microelectrode determined hydrogen concentration in mouse livers

Mouse #	H_2 Range, (μM)	Mean ± std. dev.	Sites measured
1	43-63	54 ± 9	10
2	29-89	53 ± 18	12
3	43-68	57 ± 11	12

The sites measured included all lobes of the liver, and was accomplished by insertion of a 50μmeter H_2 sensing probe into live (but anesthetized) mice. The mice were female strain C57Bl (Jackson Labs, Bar Harbor, ME) and were anesthetized with halothane. This data is part of an in press manuscript (40).

Table 2. Microelectrode determined hydrogen concentrations within a single mouse

Tissue	H_2 Range (μM)	Mean ± std. dev.	Sites measured
spleen	33, 65 (two measurements)	------	2
small intestine*	129-279	174 ± 58	7

*inside the intestinal wall

Recent Assays of H_2 oxidation activity in S. typhimurium

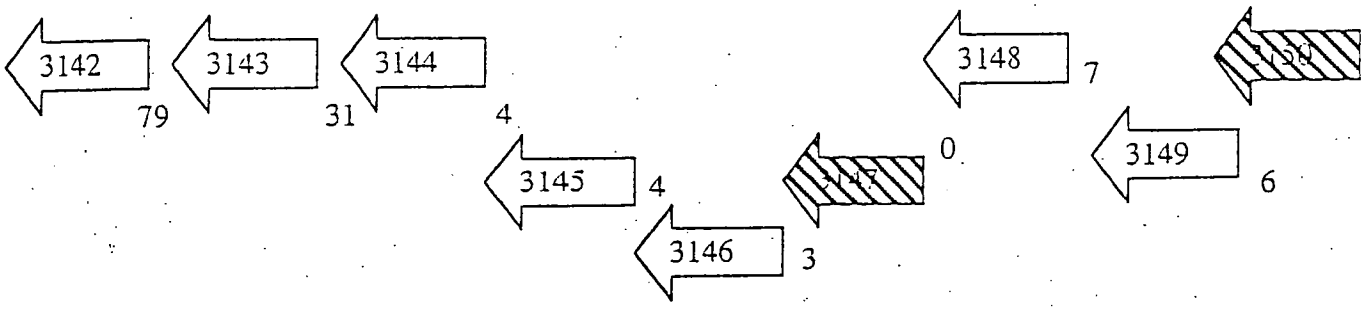
To initiate studies on use of H_2, we determined the ability of the virulent strain ATCC 14028s to express H_2 uptake activity. When grown in a rich medium and incubated with H_2 during growth, the strain had excellent H_2 oxidizing activity (about 9.5 nmoles H_2 used per 10^8 cells per min). This specific whole cell activity is 3 times more than the highest levels we ever observe in *Helicobacter pylori*, and 6--8 times the level we commonly observe in two other H_2-oxidizing bacteria (*H. hepaticus* and *B. japonicum*) by the same method and all done amperometrically in this lab. Most (about 93%) of this *S. typhimurium* activity was coupled to oxygen uptake; when the oxygen was totally consumed (monitored polarographically at the same time as H_2 uptake), H_2 uptake continued for 6-8 minutes, but at a much lesser rate than when O_2 was provided. It is likely that this H_2 oxidation could be coupled to reduction of endogenous acceptors within the cells, such as fumarate. Injection of 38 nmoles of O_2 into the (7 ml) anaerobic amperometric chamber caused H_2 uptake to resume at the high rate. To confirm that a respiratory chain was operating in H_2 oxidation, cyanide inhibition experiments were performed. Cyanide is well-known to inhibit respiratory chains that contain O_2 binding heme–proteins (i.e. cytochrome terminal oxidases). Addition of 0.1mM cyanide to the *S. typhimurium* for 10 min, prior to the start of the assay inhibited the O_2-dependent hydrogenase activity 48% whereas addition of 1.0 mM cyanide inhibited 90% of the activity compared to no inhibitor added. The cyanide addition did not inhibit the H_2 activating hydrogenase enzyme per se, as the rate of H_2 uptake with methylene blue provided as electron acceptor was unchanged by the cyanide addition. Therefore, it appears that the bulk of H_2 oxidation carried out by *S. typhimurium* is coupled to an aerobic respiratory chain. Multiple terminal oxidases that use O_2 are predicted from the genome sequences of both *S. typhimurium* and *S. typhi* (2), and intermediate electron carriers (cytochromes and quinone) are also evident from the sequence information. Still it was a surprise to see that H_2 oxidation was O_2-reduction associated as that was never reported previously for *S. typhimurium*.

D. Research Design and Methods

Generating Mutants

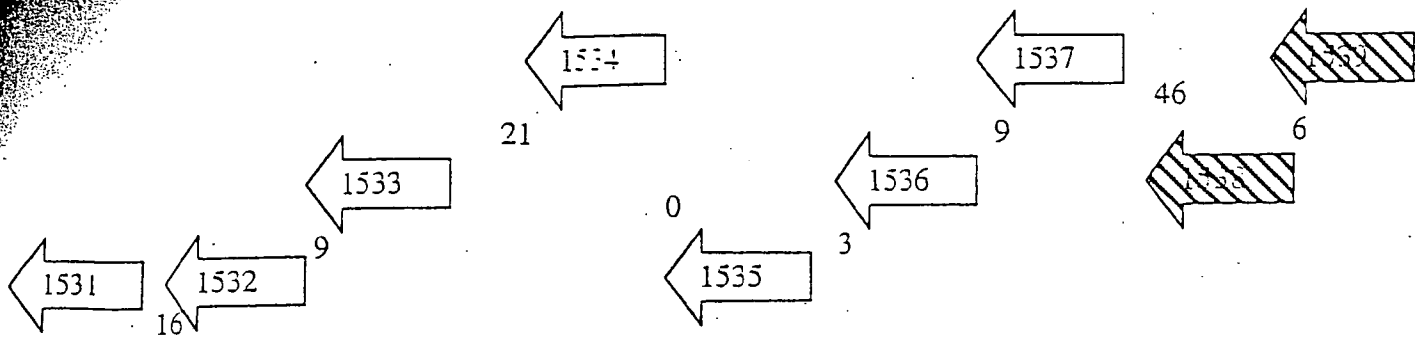
The parent strain will be ATCC 14028s, provided by Prof. John Gunn. This strain is virulent, and is readily amenable to genetic manipulations as well as to pathogenicity studies (7). The genome sequenced strain LT2 is not considered to be pathogenic, but from the sequence, we can identify 3 separate regions, each encoding a predicted H_2 uptake hydrogenase. These 3 hydrogenase-encoding (a two subunit NiFe-hydrogenase) regions are in separate operons, and each operon also contains gene(s) encoding proteins that normally couple the low potential electrons from H_2 splitting to electron carriers (heme-proteins of the heme b type) that normally funnel this energy into the respiratory chain. However, one or more of these hydrogenases may couple H_2 oxidation to organic acid reduction, but still in an energy-conserving manner (see Background and Significance). We will use primers based on these regions (the structural genes) to (sequentially) generate the targeted mutants. Our goal is to first obtain 4 separate mutants for virulence studies, one in each of the hydrogenases, and a triple mutant lacking all three. After verifying the mutation genetically, each mutant will be first tested for H_2 uptake (oxidation) activity with O_2 as the terminal acceptor (see below) to assess its ability to oxidize H_2 in a respiratory manner. The 3 regions are depicted as groups 1, 2, and 3 in three separate figures (the next 3 pages).

Group 1



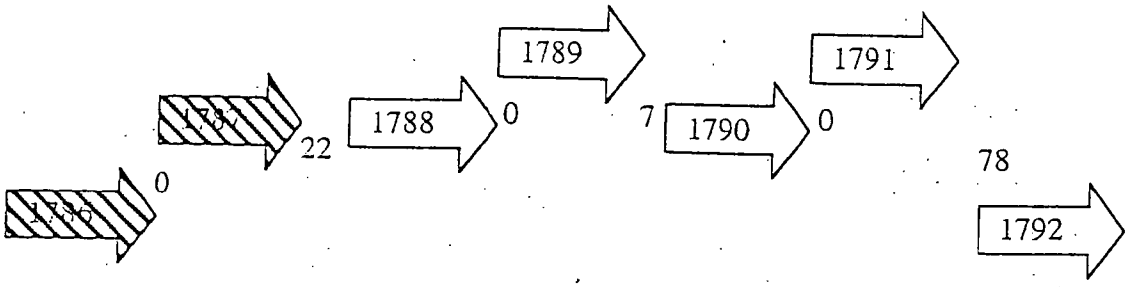
Common Name Group 1	Primary Locus Name	TIGR Locus Name	Gene Coordinates 5' to 3'
quinone reactive Ni/Fe hdrogenase, small subunit	STM 3150	NT01ST3949	3313762 to 3312647
function unknown	STM 3149	NT01ST3948	3312641 to 3311658
putative cytochrome Ni/Fe component of hydrogenase-2	STM 3148	NT01ST3947	3311665 to 3310490
hydrogenase-2, large subunit	STM 3147	NT01ST3946	3310490 to 3308790
putative processing element for hydrogenase-2	STM 3146	NT01ST3945	3308787 to 3308296
putative hydrogenase	STM 3145	NT01ST3944	3308300 to 3307815
putative hydrogenase expression/formation protein	STM 3144	NT01ST3943	3307819 to 3307481
hydrogenase-2 operon protein	STM 3143	NT01ST3942	3307450 to 3307205
putative ferrichrome-binding periplasmic protein	STM 3142	NT01ST3941	3307126 to 3306077

Group 2



Common Name Group 2	Primary Locus Name	TIGR Locus Name	Gene Coordinates 5' to 3'
putative hydrogenase-1 small subunit	STM 1539	NT01ST1902	1614904 to 1613804
putative hydrogenase-1 large subunit	STM 1538	NT01ST1901	1613798 to 1611999
putative Ni/Fe-hydrogenase 1 b-type cytochrome subunit	STM 1537	NT01ST1900	1612045 to 1611305
putative hydrogenase maturation protease	STM 1536	NT01ST1899	1611296 to 1610691
putative hydrogenase protein	STM 1535	NT01ST1898	1610688 to 1610392
putative hydrogenase	STM 1534	NT01ST1897	1610392 to 1609985
putative hydrogenase	STM 1533	NT01ST1896	1609964 to 1608906
putative dehydrogenase protein	STM 1532	NT01ST1895	1608915 to 1608031
putative hydrogenase	STM 1531	NT01ST1894	1608015 to 1607677

Group 3



Common Name Group 3	Primary Locus Name	TIGR Locus Name	Gene Coordinates 5' to 3'
hydrogenase-1 small subunit	STM 1786	NT01ST2221	1884828 to 1885943
hydrogenase-1 large subunit	STM 1787	NT01ST2222	1885943 to 1887733
putative Ni/Fe-hydrogenase 1 b-type cytochrome subunit	STM 1788	NT01ST2223	1887755 to 1888483
putative hydrogenase maturation protease	STM 1789	NT01ST2224	1888483 to 1889076
putative thiol-disulfide isomerase and thioredoxins	STM 1790	NT01ST2225	1889069 to 1889470
putative hydrogenase-1 protein	STM 1791	NT01ST2226	1889470 to 1890315
putative cytochrome oxidase, subunit I	STM 1792	NT01ST2227	1890393 to 1891934

Construction of hydrogenase gene deletions/transcriptional reporters

To begin to study the function of the genes described above, non-polar deletions will be constructed. We will utilize the lambda Red system (38) which has been used successfully by many researchers to rapidly construct deletion mutations. We have been using this system in our laboratory routinely for the last year to construct deletions. In general, one amplifies by PCR an antibiotic cassette located on a specially designed vector, and the primers used in this amplification contain homologous sequences to the gene of interest at their 5' ends (30 bp at each end). This PCR fragment is electroporated into a strain containing a temperature sensitive plasmid encoding enzymes that disallow degradation of the incoming linear DNA and allow for efficient recombination. The antibiotic cassette can be eliminated if desired to leave only a few base "scar" in place of the deleted DNA. The system can be adjusted to create non-polar or polar deletions. The deletions described below will be constructed with scar regions containing a stop codon in all 6 frames, as well as a ribosome binding site at the rightmost (5') end, which will reengage the ribosomes for downstream gene transcription. Therefore, all of the constructed deletions will be non-polar. This is necessary due to the fact that some of the hydrogenase-encoding regions have downstream genes transcribed in the same orientation, but not likely involved in hydrogenase function see figures labelled from group 1, group 2 and group 3 (hydrogenase-related genes). All deletions will be confirmed by PCR with primers designed to bind outside of the deleted region.

Dr. Jim Slauch has created a recent advancement of this system (39). He has designed plasmids carrying the *lacZ* (β-galactosidase) reporter that targets the unique "scar" left behind when the antibiotic cassette is deleted. Therefore, one can easily convert deletions into transcriptional fusions using this system. We have obtained these vectors from Dr. Slauch and have used them successfully several times in our laboratory. Therefore, β-galactosidase fusions will be constructed to all genes under study. This will enable (later) studies on regulation once we know which hydrogenases are the most important.

For the genes in group 1 (STM3142-STM3150), a deletion encompassing all of the genes involved in hydrogenase function (STM3143-3150) might be too large (6557 bp) for efficient construction by the lambda Red system. Therefore, we will design primers to delete STM3147-STM3150 (4972 bp), which includes both the hydrogenase large and small subunits. Deletions as large as 5.5 Kb have been reported as constructed by the lambda Red system, so this should be relatively easy to construct. Group 2 encompasses STM1531-1539. The small and large subunits of the hydrogenase are the first two genes of the putative operon (STM 1539 and STM 1538). These two genes will be removed, creating a 2905 bp deletion. In group 3 (STM1786-1792), we will delete genes STM1786 and STM1787 (also 2905 bp) encoding the hydrogenase small and large subunits. As mentioned above, all deletions will be non-polar so that downstream genes potentially not involved in hydrogenase maturation or function that may be co-transcribed, and are in the same orientation, remain unaffected.

Amperometric H_2 oxidation assays

We measure disappearance of H_2 directly by amperometry, in contrast to the approach sometimes used of measuring presumed H_2 loss or uptake based on dye reduction. The latter method (dye reduction) is not always specific for hydrogenase, as dehydrogenase activity may confound interpretations, and the redox dye chosen can influence the results greatly (due to their different redox potentials and degree of interactions with the enzyme). The method we use is based on reversing the polarity of a Clark type probe originally designed for measuring oxygen (18). The polarizing voltage is provided by a precisely-tuned low voltage supply (manufactured by the PI, see 19,20), and has been used to study H_2 metabolism in many different bacteria (in over 60 published studies) in the last 22 years (see 21). Typically, 40—60 nmoles of H_2 is injected into the (6 ml

volume) sealed chamber containing cells, and loss of this H_2 continuously over a period of minutes is recorded. The method is sensitive down to sub-microM levels of H_2, so binding affinity measurements can be made reliably. Oxygen is also measured by the use of a second probe also inserted into the sealed custom-made amperometric chamber. All probes are tightly sealed into the chamber by rubber gaskets prior to the start of the assays. The chamber can easily be made anaerobic by sparging it with argon, and using argon-sparged buffer as the loading medium ensures a constant anaerobic environment (monitored by the oxygen probe) if needed. Oxygen is injected through a sealed port as needed from an O_2-saturated stock solution. Currently we know that the bulk of the H_2 oxidation we measured in *S. typhimurium* ATCC 142028s is O_2-reduction coupled (see Prelim Results). When O_2 is exhausted from the amperometric chamber only 7% of the H_2 uptake activity remained (this may be H_2 oxidation coupled to reduction of residual fumarate or another organic acid within the cells). By adding cyanide, an inhibitor of heme proteins that bind O_2, the rate of H_2 oxidation was reduced greatly (see Prelim Results). This indicates that the bulk of H_2 oxidation is indeed coupled to respiration, and this likely occurs through cytochrome-dependent electron transport (energy conserving) processes. The H_2 oxidation activity of each mutant type will be carefully measured for comparison to the parent strain and to all the other mutants; this will be done both with O_2 (the complete respiratory chain) and with methylene blue (accepts electrons directly from the H_2 splitting hydrogenases enzyme, the first step in H_2 oxidation) as electron acceptor. This will give an indication as to the respiratory connection of that particular hydrogenase. For the latter experiments, the cells will be treated for 10 min with a small amount of detergent to partially permeabilize them to the redox dye (22). If one of the hydrogenases is important for virulence (based on inoculation of the mutant strains into mice), but does not appear to be coupled to O_2-dependent respiration, then that activity will be characterized. This will involve studying electron acceptor specificity (14,23), including physiological acceptors such as formate, fumarate, or quinones (24). Such studies will be done with membrane preparations or crude extracts. It is expected that the hydrogenases studied here will be either periplasmic or membrane associated (13). An anaerobic respiratory H_2 oxidizing enzyme could still be coupled to proton translocation and therefore to energy conserving reactions (see 12 and 13), but the bulk of the H_2 oxidation we have observed in *S. typhimurium* was O_2 reduction coupled, so it is anticipated that a hydrogenase in the family of membrane-bound NiFe type of O_2-dependent respiratory hydrogenase (25) will be of the most interest. Another role for some of the (sequence-predicted) two subunit hydrogenases may be as sensors of H_2, to permit synthesis of the H_2 binding/oxidizing enzymes (see 13 and 25).

Affinity for H_2

By measuring the affinity of *H. pylori* for H_2 we concluded the enzyme is most often saturated with the substrate while the bacteria are residing within animal tissues, specifically in the mucosal lining of the stomach. By knowing the whole cell H_2 binding affinities of the various (*S. typhimurium*) hydrogenase mutant strains combined with knowing their virulence characteristics, we can associate certain hydrogenases with virulence and H_2 affinities. The binding affinities for H_2 will be determined by the wild type and mutant strains by assaying H_2 oxidation at a variety of H_2 levels, from saturation down to limiting levels in the μM range as described (3,26). By amperometry this can be done easily, and levels as low as 50nM can be measured (20,32,34). In this way we can understand the range of H_2 concentrations in the tissue that would be predicted to be most important for growth/survival in the host. These affinities will be interpreted with the knowledge of the H_2 levels we measure in the live animal (see Preliminary Results). When growth of pathogenic bacteria on molecular hydrogen within animals is understood in more detail, the

proposed studies may lead to studies by nutritional experts and others on the effects of diet on the H_2 producing reactions occurring in the large intestine of animals.

Virulence Determination

Mice infected with *S. typhimurium* exhibit typhoid fever-like symptoms, and even the intestinal and extraintestinal lesions closely resemble those observed in (human) typhoid fever victims (4). Mice develop elevated temperature (as indicated by ruffled fur) between 4 and 8 days after oral infection, but they do not develop diarrhea. In mice, the bacteria spread systemically with severe pathological changes and high bacterial numbers overwhelming the liver, spleen, and mesenteric lymph nodes and Peyers patches. Animal studies are modeled after the ones described by Gunn et al. (see 7). Female BALB/c mice will be inoculated orally with overnight cultures of *S. typhimurium* that were washed and diluted in PBS. Approximately 10^6 CFU's will be inoculated into each mouse. This is one log unit above the LD_{50}, and has been a useful inoculant level for similar studies of virulence factors (7). As another approach, animals will also be inoculated intraperitoneally with a much lower inoculum level (7). The average days of survival and numbers of surviving mice will be recorded and compared. It is anticipated these studies will proceed for approximately 40 days. Also, mixtures of strains (two strains per mouse, inoculated orally) will be assayed in competition-type studies as described (7,27) on the mutant strains that show a virulence deficiency from the initial (single inoculant) studies. The wild type strain together with each mutant type will be inoculated and the competitive index determined based on the recovery of colony forming units of the two inoculated types. The liver and spleen will be removed from moribund mice and the organs homogenized, diluted and plated. Each strain will be antibiotic resistance marked for this assay. It is also important to perform a wild type versus wild type competition assay (with the two different wild types differing only in antibiotic resistance) to ensure they are equitably recovered from the homogenized organs. Also, in vitro competitions where the two strains are grown in LB medium (but without H_2 provided) will be important to ensure that any in vivo affects are not due to growth differences in the two test strains (see 7). The virulence determination experiments will be done in the PI's lab, but with advice as needed by Dr. Gunn. Dr. Gunn's lab has previously performed all of the procedures anticipated to be necessary for the virulence studies, and personnel from the PI's lab are expected to be in close contact with them.

Hydrogen Reduction of Cytochromes

The roles of the various hydrogenases in respiratory metabolism, in particular for heme-protein mediated respiratory chains, will be studied by use of membrane particles obtained from the mutants. As the PI's lab has done on many types of H_2 oxidizing bacteria (see 21), absorption difference spectral experiments with H_2 as the reductant will be performed. The membrane particles will be prepared as described (23,27), in the presence of the antioxidant butylated hydroperoxide (23) to maximize recovery of H_2 oxidation respiratory activity. The washed membrane samples will be oxidized with O_2 by air exposure (28), and the spectra recorded (i.e. memorized by the instrument). The same sample will be sparged with argon in order to remove O_2 and then the sample will be reduced with H_2; the new spectrum will be recorded and the O_2 spectrum subtracted to reveal those heme components that are H_2 reduced (27,29). This will begin to give an indication as to which H_2 using enzymes are coupled to an respiratory energy generating system, and should be in agreement with our cyanide inhibition data (see Preliminary Results). Performing the difference absorption spectra with sodium dithionite as the reductant will permit a comparison of which heme components are specifically reduced by H_2 versus the total complement that are available in the membranes (27-30). Also, difference spectral absorptions done with cyanide present (27,29) will reveal the type of terminal oxidases used by the putative H_2 respiration

pathway(s). It is known that H_2 oxidizing chains can sometimes involve different terminal oxidases than those used by other reducing substrates (like by NADH or succinate, for example). The electron transport factors responsible for energy generation specifically via electrons from H_2 are desired for the time being. It is anticipated that pursuing aims #1 and #2 together will reveal that the hydrogenase(s) that are coupled to a respiratory chain are also the ones that are inhibited by cyanide, and the most important for growth in the animal. However, as pointed out earlier, a hydrogenase that is not coupled to O_2 reduction could still prove to be important in virulence. If this is so, that enzyme will be studied for its ability to reduce fumarate or other potential electron acceptors. This will be done for membranes or extracts containing periplasmic components if needed (see 13) from the strain containing only that hydrogenase of interest.

Generation of some double mutants (lacking two of the three hydrogenases) will likely be needed to create the starting material for making membrane preparations for some experiments. This is so that the results on H_2 mediated cytochrome reduction can be attributed to a particular hydrogenase. However, we can't propose which hydrogenases are the important players as yet. The PI's lab has extensive experience in generating and studying H_2 oxidizing membrane preparations from many different bacteria (see 21).

Begin Preliminary Regulation Studies (if time permits)

Hydrogenases are oftentimes regulated at the transcriptional level by O_2, H_2, and by nickel (13,25). As the mutant strains here can be easily converted into transcriptional fusions (see above) we may begin studies on the roles of the above regulators on the transcription of hydrogenase(s) that are first shown to be important in virulence. These regulators (H_2, O_2, nickel) have been studied extensively for their effect on hydrogenase expression in (H_2-oxidizing) bacteria in the PI's lab (see 21). For studying nickel-dependence, medium will have to be first treated to remove the metal and then high-purity metal salts added back to the medium (22). Extensive regulation experiments may have to await inclusion in an R01 proposal in a few years.

E. Human Subjects

Non-applicable

F. Vertebrate Animals

For initial testing of the four mutant types plus the parent strain for virulence over a 40 day period, it is anticipated that 80 (BALB/c) mice will be used the first year. This number will permit use of 8 mice for each of the 5 strains (4 are mutant strains) plus a replicate of the entire experiment. These mice will be infected by oral gavage of $1x10^6$ live bacteria into the stomach. When we know which hydrogenases are the most important, intraperitoneal and mixed inoculation experiments (see Methods) will be done to rigorously assess the virulence of these strains. This may require up to 80-90 additional mice (year 2). Harvesting of organs from moribund mice will be done for the mixed inoculation experiments (see Methods). If mice need to be euthanized this will be done by CO_2 overdose as recommended by IACUC. University of Georgia Animal Welfare Assurance no. A3437-01 approved 03-27-02.

G. Literature Cited

1. Salyers, A.A., and D. Whitt. 2002. Bacterial Pathogenesis, ASM Press, Washington, D.C.
2. See TIGR site at www.tigr.org/tdb.
3. Olson, J.W. and R.J. Maier. 2002. Molecular hydrogen as an energy source for *Helicobacter pylori*. Science **298**:1788-1790.

4. Zhang, S., Kingsley, R.A., Santos, R.L., Andrews-Polymenis, H., Raffatellu, M., Figueiredo, J., Tsolis, R.M., Adams, L.G., and A.J. Baumler. 2003. Molecular pathogenesis of *Salmonella enterica* serotype typhimurium-induced diarrhea. Infect. Immun. **71**:1-12.
5. Boyer, E., Bergevin, I., Malo, D., Gros, P., M.F.M. Cellier. 2002. Acquition of Mn(II) in addition to Fe(II) is required for full virulence of *Salmonella enterica* serovar typhimurium. Infect. Immun. **70**:6032-6042.
6. Santos, R.L., Zhang, S., Tsolis, R.M., Kingsley, R.A., Adams, L. G., and A.J. Bäumler. 2001. Animal models of Salmonella infections: enteritis versus typhoid fever. Micro. Infect. **3**:1335-1344.
7. Gunn, J.S., Ryan, S.S., Van velkinburgh, J.C., Ernst, R.K. and S.I. Miller. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium. Infect. Immun. **68**:6139-6146.
8. Sawers, R.G., Jamieson, D.J., Higgins, C.F., and D.H. Boxer. 1986. Characterization and physiological roles of membrane-bound hydrogenase isoenzymes from *Salmonella typhimurium*. J. Bacteriol. **168**:398-404.
9. Jamieson, D.J., Sawers, R.G., Rugman, P.A., Boxer, D.H., and C.F. Higgins. 1986. Effects of anaerobic regulatory mutations and catabolite repression on regulation of hydrogen metabolism and hydrogenase isoenzyme composition in *Salmonella typhimurium*. J. Bacteriol. **168**:405-411.
10. Jones, R.W. 1980. The role of the membrane-bound hydrogenase in the energy-conserving oxidation of molecular hydrogen by *Escherichia coli*. Biochem. J. **188**:345-350.
11. Adams, M.W.W., Mortensen, L.E., and J.S. Chen. 1981. Hydrogenase. Biochim. Biophys. Acta. **594**:105-176.
12. Friedrich, B., and E. Schwartz. 1993. Molecular biology of hydrogen utilization in aerobic chemolithotrophs. Annu. Rev. Microbiol. **47**:351-383.
13. Vignais, P.M., Billoud, B., and J. Meyer. 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. **25**:455-501.
14. Maier, R.J., C. Fu, J. Gilbert, F. Moshiri, J. Olson, and A.G. Plaut. 1996. Hydrogen uptake hydrogenase in *Helicobacter pylori*. FEMS Microbiology Letters **141**:71-77.
15. Wolin, M.J., and T.L. Miller. 1994. Acetogenesis from CO_2 in the Human Colonic Ecosystem, in Acetogenesis, Drake, HL, Ed. Chapman & Hall, New York, 365-385.
16. Bond, J.H. and M.D. Levitt and R. Prentiss. 1975. Investigation of small bowel transit time in man utilizing pulmonary hydrogen (H_2) measurements. J. Lab. Clin. Med. **85**:546-555.
17. Olesen, M., and E. Gudmand-Hoyer. 1997. Maldigestion and colonic fermentation of wheat bread in humans and the influence of dietary fat. Am. J. Clin. Nutr. **66**:62-66.
18. Wang, R.T. Amperometric Hydrogen Electrode. Methods. Enzymol. **69**:409-413.
19. Maier, R.J., and S.S. Hom. 1985. Hydrogen uptake negative mutants of Rhizobium and their use in the isolation of *hup* genes. Methods Enzymol. **118**:528-537 (and references therein).
20. Ferber, D.M., B. Moy, and R.J. Maier. 1995. *Bradyrhizobium japonicum* hydrogen-ubiquinone oxidoreductase activity: quinone specificity, inhibition by quinone analogs, and evidence for separate sites of electron acceptor reactivity. BBA **1229**:334-346.
21. Community of Science website for the PI, at http://expertise.cos.com/cgi-bin/indexes.
22. Stults, L.W., E.B. O'Hara, and R.J. Maier. 1984. Nickel is a component of hydrogenase in *Rhizobium japonicum*. J. Bacteriol. **159**:153-158.
23. Mutaftschiev, S., M.R. O'Brian, and R.J. Maier. 1983. Hydrogen oxidation activity in membranes from *Rhizobium japonicum*. Biochim. Biophys. Acta. **722**:372-380.

24. Ferber, D.M., and R.J. Maier. 1993. Hydrogen-ubiquinone oxidoreductase activity by the *Bradyrhizobium japonicum* membrane bound hydrogenase. FEMS Microbiol. Lett **110**:257-264.
25. Maier, R.J., Mehta, N.S., and Olson, J.W. 2002. Genes and proteins involved in nickel dependent hydrogenase expression. *In: Physiology and Biochemistry of Anaerobic Bacteria.* Springer-Verlag, New York.
26. McCrae, R.E., Hanus, J., and H.J. Evans. 1978. Properties of the hydrogenase system in *Rhizobium japonicum* bacteroids. Biochem. Biophys. Res. Commun. **80**:384-390.
27. O'Brian, M.R., and R.J. Maier. 1983. Involvement of cytochromes and a flavoprotein in hydrogen oxidation in *Rhizobium japonicum* bacteroids. J. Bacteriol. **155**:481-487.
28. Keefe, R.G., and R.J. Maier. 1993. Purification and characterization of an O_2-utilizing cytochrome-*c* oxidase complex from *Bradyrhizobium japonicum*. Biochim. Biophys Acta. **1183**:91-104.
29. Wong, T-Y, and R.J. Maier. 1984. Hydrogen-oxidizing electron transport components in nitrogen-fixing *Azotobacter vinelandii*. J. Bacteriol. **159**:348-352.
30. Pihl, T., L.K. Black, B.A. Schulman, and R.J. Maier. 1992. Hydrogen-oxidizing electron transport components in the hyperthermophilic archaebacterium *Pyrodictium brockii*. J. Bacteriol. **174**:137-143.
31. Kolonay, J.F., F. Moshiri, R.B. Gennis, T.M. Kaysser, and R.J. Maier. 1994. Purification and characterization of the cytochrome *bd* complex from *Azotobacter vinelandii*: Comparison to the complex from *Escherichia coli*. J. Bacteriol. **176**:4177-4181.
32. Olson, J.W., Fu, C. and R.J. Maier. 1997. The HypB protein from *Bradyrhizobium japonicum* can store nickel and is required for the nickel-dependent transcriptional regulation of hydrogenase. Mol. Microbiol. **24**:119-128.
33. Brown, N.J., Rumsey, R.D. and N.W. Read. 1987. Adaption of hydrogen analysis to measure stomach to caecum transit time in the rat. Gut **28**:849-854.
34. Black, L., Fu, C. and R.J. Maier. 1994. Sequences and characterization of *hupU* and *hupV* genes of *Bradyrhizobium japonicum* encoding a possible nickel-sensing complex involved in hydrogenase expression. J. Bacteriol. **176**:7102-7108.
35. Laurinavichene, T.V., Zorin, N.A., and Tsygankov, A.A. 2002. Effect of redox potential on activity of hydrogenase 1 and hydrogenase 2 in *Escherichia coli*. Arch. Microbiol. **178**:437-442.
36. Sawers, R.G., Ballantine, S.P., and Boxer, D.H. 1985. Differential expression of hydrogenase isoenzymes in *Escherichia coli* K-12: evidence for a third isoenzyme. J. Bacteriol. **164**:1324-1331.
37. Andrews, S.C., Berks, B.C., McClay, J., Ambler, A., Quail, M.A., Golby, P., and Guest, J.R. 1997. A 12-cistron *Escherichia coli* operon (hyf) encoding a putative proton-translocating formate hydrogenlyase system. Microbiology **143**:3633-3647.
38. Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U.S.A **97**:6640-6645.
39. Ellermeier, C. D., A. Janakiraman, and J. M. Slauch. 2002. Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290**:153-161.
40. Maier, R.J., J. Olson, and A. Olczak. 2003. Hydrogen oxidizing capabilities of *Helicobacter hepaticus* and *in vivo* availability of the substrate. J. Bacteriol. in press.

Hydrogen Use by Enteropathogenic Bacteria is Critical for Virulence

R.J. Maier*, A. Olczak*, S. Maier*, S. Soni#, and J. Gunn#
*Dept of Microbiology, Univ. of Georgia, Athens GA 30602
Corresponding author: rmaier@uga.edu tel 706 542 2323 fax: 542 6874

Department of Molecular Virology, Immunology and Medical Genetics; Department of Medicine, Division of Infectious Diseases; and The Center for Microbial Interface Biology, The Ohio State University, Columbus, OH 43210 Ohio State University, Columbus, OH

Abstract. Based on available annotated gene sequence information the enteric pathogen *Salmonella,* like other enteric bacteria, contains three putative membrane-associated H_2-using hydrogenase enzymes. Here we show that H_2 oxidation by the parent strain is coupled to respiration and the 3 distinct hydrogenases are each able to use H_2 in the respiratory pathway to oxygen. By use a microelectrode probe, we detected H_2 within (mouse) tissues that are colonized by the bacterium. The low potential reductant H_2 was measured in the intestinal tract and in the liver/spleen tissue at about 80 and 20 times, respectively that of the half-saturation affinity of the whole cells for H_2. All 3 hydrogenase enzymes contribute to virulence of the bacterium in a typhoid fever-mouse model. The combined removal of all 3 hydrogenases results in a strain that is avirulent and (in contrast to the parent strain) that is unable to pass the intestinal tract to invade liver or spleen tissue. Therefore, H_2 utilization is required for energy production to permit *Salmonella* growth and subsequent virulence during infection.

Together enteric pathogens are responsible for an estimated 2 million deaths annually (4, and world health organization site, see www.who.int/health-topics/index.html), and cause millions more cases of diarrheal illness annually, even in developed countries (see center for disease control, www.cdc.gov/health/default.htm). Based on annotated whole genome sequences, intestinal disease-causing bacteria such as *Salmonella*, *Escherichia coli*, *Shigella*, *Yersinia*, and *Campylobacter* all contain homologous hydrogenases (2). These hydrogenases typically split molecular H_2 via a unique NiFe metal center, with the release of protons and low potential electrons. The NiFe hydrogenase enzymes are membrane associated where the electrons can be sequentially passed to heme-containing or quinone-reactive proteins. It was suggested that H_2 using hydrogenase enzymes might enable enteric bacteria to glean energy from the splitting of molecular hydrogen (1). The high-energy gas is produced by colonic flora within animals (2) and because it is freely diffusible, the gas can be measured within both intestinal and non-intestinal tissue (2,3). The gastric pathogen *Helicobacter pylori* contains only a single membrane associated hydrogenase, and it was demonstrated that use of H_2 by this enzyme is important for the bacterium's ability to colonize the stomach (3). Here we address the importance of H_2 use to the pathogenicity of *Salmonella typhimurium*, a common food poisoning bacterium closely related to the typhoid fever-causing bacterium *S typhi*; we assess the role of enteric H_2 use in the mouse model of typhoid fever (5).

Methods

Amperometric Hydrogenase assays. The conditions for obtaining hydrogenase activity involved growing cells on a blood-containing medium (6), in a microaerobic H_2

containing atmosphere (see table 1 legend). *S. typhimurium* cells grown one day on the BA plates were suspended in PBS, and 8 ml samples at cell concentrations of 8 x 10^8 cells per ml were assayed for H_2 and O_2uptake activities simultaneously. This was accomplished on the same sample in a stirred and sealed amperometric chamber (7). Hydrogen and oxygen were added as needed from gas-saturated solutions of phosphate buffered saline. H_2 uptake rates were linear until the substrate reached levels about 3--4 μM. For methylene blue dependent rates, the chamber lacked oxygen but contained MB at 200 μM, and the cells were permeabilized with TritonX-100 (8) before assay. Cell numbers were determined by performing dilutions and plate counts on MacConkey medium.

Hydrogen levels in tissues. Measurements of H_2 levels within the small intestine of the mice were performed by making a small incision into the intestinal wall with a razor blade, and inserting a 50 μmeter tip size H_2 microelectrode probe less than 0.5 mm into the intestine. For splenic H_2 determinations, the probe was placed from 0.2 to less than 1.0 mm into the spleen tissue as described previously for our H_2 measurements in liver tissues of live mice (8). These determinations (including instrument calibrations) were performed like those described in detail previously for our H_2 measurements in other tissues (3,8). Care was taken to keep all the organs as intact as possible during surgery and microelectrode measurements, and the mouse was kept under anesthetic for the procedure. Twelve independent measurements were made (four each on 3 separate mice) for each tissue and the mean of these is reported.

Mutant strain construction. All mutations were in the structural genes for hydrogenase and were constructed in a way so as not to disrupt downstream genes. The three hydrogenase genes targeted are ones that are homologous to genes encoding membrane-associated NiFe uptake hydrogenases (a fourth putative hydrogenase in *S. typhimurium* is homologous to the hydrogenase termed HycE of *E. coli* that is proposed to be associated with electron transfer reactions within a formate hydrogen lyase complex, see 1). *S. typhimurium* background strain ATCC1402s (JSG210) was used as the parent for the construction of all the mutants. The Lambda Red system was used to construct deletion mutations in the hydrogenase genes (9). An antibiotic cassette located on plasmid pKD4 was amplified by PCR. Primers were designed (at their 5' ends) to contain homologous sequences to the DNA outside the fragment assigned for deletion. Primers were designed to delete a 4972 bp region of group 1 genes (STM 3147 through STM 3150) represented by coordinates 3313762 to 3308790 in the TIGR comprehensive microbial resource for *S. typhimurium*. Group 2 gene deletion included genes STM1538 and STM 1539 by creating a 2905 bp deletion fragment (coordinates1614904 to 1611999 deleted). For group 3, a deletion of 2905 bp was made (coordinates 1884828 to 1887733 which includes genes STM 1786 and STM 1787). Genes within the deleted regions encoded hydrogenase large and small subunits. Each of the PCR fragments were transformed by electroporation into a strain of *Salmonella typhimurium* containing the Red helper plasmid allowing uptake of linear DNA and recombination. The antibiotic resistance cassette in the mutants was eliminated by transforming the strains with the FLP synthesis inducing plasmid, pCP20 (9). FRT-flanked resistance genes, as well as FLP helper temperature sensitive plasmid, were both lost at 43°C. Double mutants were

obtained by P22 HT*int*-mediated transduction of an antibiotic marked single gene deletion strain into the appropriate single gene deletion strain by antibiotic selection, followed by transformation with the pCP20 plasmid and elimination of the antibiotic cassette. The triple mutant was constructed in a similar manner using the appropriate double deletion mutant (genes I and 2 negative) as the recipient. All deletions were confirmed by PCR with primers designed outside of the deleted DNA regions.

Each deletion left a few base "scar" in place of deleted DNA. The scar region contains a ribosome binding site at the 5' end as well as a start codon, which will allow reengagement of the ribosome for the downstream gene transcription. Also, rtPCR of genes directly downstream of the deletion (for group 1, STM 3145, for group 2, STM1536, and for group 3, STM1789) was performed in order to confirm the lack of polar affects. Additionally, expression of STM 3142 (ferrichrome-binding periplasmic protein) was measured by rtPCR in the triple mutant strain and was found to be the same as for the wild-type strain. Therefore, the expression of genes downstream of the deleted regions was unaffected, so the results presented are due to hydrogenase deficiencies only.

Mouse inoculations. (BALB/c female mice) were inoculated orally as described previously (10) with 0.1ml amounts of washed cells (containing 1 x 10^6 bacteria) suspended in PBS. Mice were observed twice daily and morbidity recorded. The organ burdens of bacteria post-inoculation were obtained by euthanizing mice (96 hr after inoculation). The liver and spleen were immediately removed from the euthanized mice, and the organs were homogenized (in PBS). Dilutions were plated onto MacConkey

agar, a medium selective for enterics, and colonies were counted the next day. No colonies were observed from homogenized organs from uninoculated mice.

Results and Discussion

Respiratory H_2 oxidation. *S. typhimurium* hydrogenase activity has been ascribed to at least two distinct but similar membrane associated hydrogenases (11,12), and possible roles for these enzymes in anaerobic energy metabolism were proposed (1). The complete genome sequence of *S. typhimurium* LT2 indicates the bacterium contains genes for three putative homologous membrane-associated H_2 utilizing type hydrogenases (the Institute for Genomic Research, www.tigr.org/tigerscripts/CMR2/genomepage3.spl?database=ntst01). The gene annotated sequence also reveals that *S. typhimurium* has several O_2 binding oxidases that could perhaps allow for the complete respiratory oxidation of electrons from H_2 all the way to O_2 reduction. If the reductant H_2 could be used simultaneously with O_2 as the acceptor (i.e. via respiration), then it is expected that a high efficiency energy yield would be available to allow H_2 mediated growth of cells (13). Therefore, we measured H_2 oxidation coupled to O_2 dependent respiration in the parent strain in various gas atmospheres and culture medium conditions, including Blood agar and microaerobic atmospheres (table 1). Previous enteric bacteria hydrogenase studies used cells grown on either a glucose-peptone medium (11) or on LB medium (12) under strictly anaerobic conditions. H_2 oxidation was monitored herein simultaneously with (O_2-dependent) respiration by use of H_2 and O_2 electrodes on the same (sealed and stirring) samples. The parent strain was able to readily oxidize H_2 at rates we observed for another H_2 oxidizing

pathogenic bacterium under the same incubation conditions (i.e. Blood agar plus a microaerobic H_2 containing atmosphere (3). Activities on Blood Agar were 4 times that on Luria Broth when both were incubated with anaerobic gas mix (compare no. 1 with no. 6). Oxygen repressed hydrogenase expression, as seen by comparing conditions 2 or 4 with no. 1 (also compare condition 7 or 9 with no. 6). This O_2 repression phenomenon on hydrogenase expression is common for respiratory hydrogenases (13). Also, incubation with H_2 augmented expression, as seen by comparing condition no. 1 with 5, and no. 6 with 10.

Additional Characteristics of H_2 Oxidation. Regarding the H_2 oxidation observed for the wild type, when O_2 was exhausted, H_2 uptake continued at a slow rate (approximately 8% of the aerobic rate) for about 7--10 min, and then (over the next 3—4 min) H_2 uptake diminished and ceased entirely. We attribute the "anaerobic" H_2 oxidation to endogenous acceptors (perhaps organic acids like fumarate) still present within the bacterium, or residual electron acceptors in the medium. This was supported by the observation that incubation of cell suspensions (cells removed from the blood agar medium into PBS) incubated in an H_2-containing atmosphere for 10-30 min at room temperature resulted in cells that no longer exhibited the anaerobic H_2 respiration activity (i.e. terminal substrate exhausted). Normally cells were suspended in PBS and assayed immediately, so a low rate of H_2 oxidation occurred without O_2. By performing hydrogenase assays in the absence of oxygen, along with use of mutant strains (see below) we conclude that some, but not all, of the endogenous or anaerobic activity (which is the minor H_2 respiration activity) could be assigned to function of hydrogenase number 1 (gene group 1), but hydrogenase number 1 is also responsible for O_2 dependent H_2 oxidation/respiration

(table 1). After the anaerobic rate ceased, and O_2 supplied again, the stoichiometry of H_2 uptake to O_2 uptake was 2.0, as expected for the complete oxidation of H_2 by O_2. That the bulk of H_2 oxidation (even in the first 5 min of the assay) occurs via respiration to O_2 was corroborated by cyanide inhibition experiments as follows. Addition of 0.1mM cyanide to the *S. typhimurium* cell suspension prior to the start of the H_2 uptake assay (15 min incubation with sodium cyanide in an argon-sparged atmosphere) inhibited 52% of the hydrogenase activity compared to the no inhibitor activity, and addition of 1.0mM cyanide inhibited 90% (to 10% of the no inhibitor added) of the H_2 uptake activity. The cyanide additions did not affect the methylene blue dependent H_2 uptake activity (i.e. the H_2 splitting hydrogenase reaction), so the inhibitor must be acting at the level of the O_2 binding heme-containing proteins (as expected).

Hydrogenases that consume molecular H_2 typically have high affinities for the substrate. By performing H_2 uptake assays amperometrically (with O_2 as the terminal acceptor) in limiting H_2 levels we determined a half saturation affinity for H_2 by wild type *S. typhimurium* to be 2.1 μM.

Characteristics of Mutant strains. Individual single mutant strains in each of the 3 hydrogenases all had decreased O_2 dependent H_2 uptake activity compared to the parent strain (table 2); this indicates that each of the 3 enzymes contribute to respiratory H_2 oxidation. Still, one of the three enzymes (encoded in gene group 1) is a lesser contributor to the overall activity (in laboratory conditions) compared to the other two hydrogenases. All double mutant combinations showed further reduced activity compared to the parent or the single mutant strains. Only the mutant strain lacking all 3

hydrogenases failed to oxidize H_2. The growth rate in LB liquid medium was the same for wild type and the triple mutant (data not shown).

To assess the ability of the strains to cause disease, a common mouse model was used (10). All double mutant strain combinations were either as virulent as the parent strain (see figure), or (for the strains containing only hydrogenase II or III) almost as virulent as the parent, so the presence of any one of the three hydrogenases is sufficient for the bacterium to cause severe disease. The expression levels of the 3 hydrogenase enzymes within the animal is not known, but due to the *in vivo* results it is clear that the hydrogenase of group 1, a minor contributor to the overall lab-grown activity, is an important enzyme for virulence. Nevertheless, the importance of hydrogenases II and III are shown by the result that a hydrogenase I mutant had the same virulence capacity as the wild type (data not shown). All double mutant strain combinations were tested in the animal, and the results confirmed that all 3 enzymes are individually sufficient for virulence (i.e. at least 50% of inoculated mice were dead at day 11 post-inoculation for all double mutant strains). However, the triple mutant lacking all H_2 oxidizing ability was dramatically less virulent; indeed out of 30 inoculated mice with that strain, none died.

Dissemination and organ burden. Liver and spleen colonization numbers by the triple mutant and the wild type were determined five days post inoculation with the result that viable *S. typhimurium* were recovered from the organs of mice inoculated with the parent (H_2-using) strain, but no cells were recovered from mice inoculated with the triple mutant. The range of colonization numbers (*S. typhimurium* recovered 96 hrs after inoculation) among 4 mice inoculated with the wild type ranged from 5.0×10^4 to 1.9×10^5 CFU per liver, and 3.0×10^4 up to 1.8×10^5 CFU per spleen. Therefore it is likely

that the mutant strain is eliminated from the intestine or during transit to the mesenteric lymph nodes.

It was proposed that H_2 produced by colonic bacteria may reach tissues within animals by a combination of cross-epithelial diffusion and vascular-based transport processes (2, 3). Molecular hydrogen levels ranged from 118 up to 239 μM in the small intestine of live mice (the mean value for 12 determinations was 168 μM), and spleen tissue H_2 levels were similar (approximately 43μM) to that we reported previously for liver tissue (8). In either case, these H_2 levels are higher than the amount needed to essentially saturate whole cell hydrogenase, based on affinities of the bacteria for H_2 when grown in the lab. For intestine and liver/spleen the measured levels were about 80 and 20 fold respectively, above the half saturation value of the cells of about 2μM (see above). We should thus expect rapid turnover of the H_2 using hydrogenases.

Conclusion. Based on our knowledge of H_2 respiration (13), the use of H_2 in an O_2 dependent respiratory pathway by *Salmonella* would be expected to result in ATP production to bolster cell growth. The animal results here demonstrate the importance of H_2 use by an enteric bacterium for survival/growth in vivo. It is likely that this is a common mechanism of energy generation by enteric pathogens within the host. The reason for some of the enteric bacteria having 3 similar (but all active) H_2 using enzymes is unknown, but could be related to different environments they encounter. The identification of agents that selectively inhibit bacterial hydrogenases (with their unique active centers containing Ni, Fe, CN, and CO), may represent potential therapeutic strategies for the elimination of *salmonella*-based and other enteric infections.

1. Bock, A., and Sawers, G. in *Escherichia coli and Salmonella typhimurium*, chapt. 18, see www.ecosal.org/ecosal/chapters/index.jsp?018. (ASM Press, Washington, D.C 2002), F.C. Neidhardt (and 9 others as editors).

2. Maier, R.J. Microbes Infect. (2003) **12**,1159-1163.

3. Olson, J., and Maier, R.J. Science. (2002) **298**,1788-1790.

4. de Bruyn, G. West J. Med. (2000) **172**,409-412.

5. Salyers, A., Whitt, D.D., Bacterial Pathogenesis: A Molecular Approach (ASM Press, Washington, D.C. 2002).

6. Olczak, A., Olson, J., and Maier, R.J., J. Bacteriol. (2002) **184**, 3186-3193.

7. Merberg, D., O'Hara, E.B., Maier, R.J., J. Bacteriol. (1986) **156**, 1236-1242.

8. Maier, R.J., Olson J., Olczak. A., J. Bacteriol. (2003) **185**, 2680-2682.

9. Datsenko, K.A., and Wanner, B.L., Proc Natl Acad Sci. USA, (2000) **97**, 6640-6645.

10. Tamayo, R., Ryan S.S., McCoy, A.J., Gunn, J.G., Infect. Immun. (2002) **70**, 6770-6678.

11. Sawers, R.G., Jamieson, D.J., Higgins, C.F., Boxer, D.H., J. Bacteriol. (1986) **168**, 398-404.

12. D.J. Jamieson, R.G. Sawers, P.A. Rugman, D.H. Boxer, C.F. Higgins. J. Bacteriol. (1986) **168**, 405-411.

13. Vignais, P.M., Billoud, B., Meyer, J., (2001) FEMS Microbiol. Rev. **25**:455-501.

Table 1. Variations of Growth Conditions for Obtaining Respiratory H_2 Oxidizing Activity

Condition no.	Description		Activity
	Medium	Gas atmosphere	(nmoles H_2/min/10^9 cells)
1	Blood Agar	Anaerobic Mix	11.9 ± 1.5
2	Blood Agar	Anaerobic Mix but 2% O_2	3.2 ± 0.4
3	Blood Agar	Campypak system	2.1 ± 0.3
4	Blood Agar	Anaerobic Mix, but 8% O_2	<0.2
5	Blood Agar	Anaerobic mix but without H_2	7.2 ± 1.2
6	Luria Broth	Anaerobic mix	2.8 ± 0.4
7	Luria Broth	Anaerobic mix, but 2% O_2	<0.2
8	Luria Broth	Campypak system	1.2 ± 0.1
9	Luria Broth	Anaerobic mix, but 8% O_2	<0.2
10	Luria Broth	Anaerobic mix, but no H_2	1.3 ± 0.3

*Anaerobic mix consists of 10% H_2, 5% CO_2, balance N_2. After sparging with this mixture, O_2 levels were below 0.2% partial pressure, but were not anaerobic. Results are mean $\pm$ std. dev. for 5 replicate independent samples. BA = Blood Agar LB = Luria Broth Campypak is a H_2 and CO_2 generating system that depletes O_2; initially the atmosphere is air, but less than atmospheric O_2 is achieved.

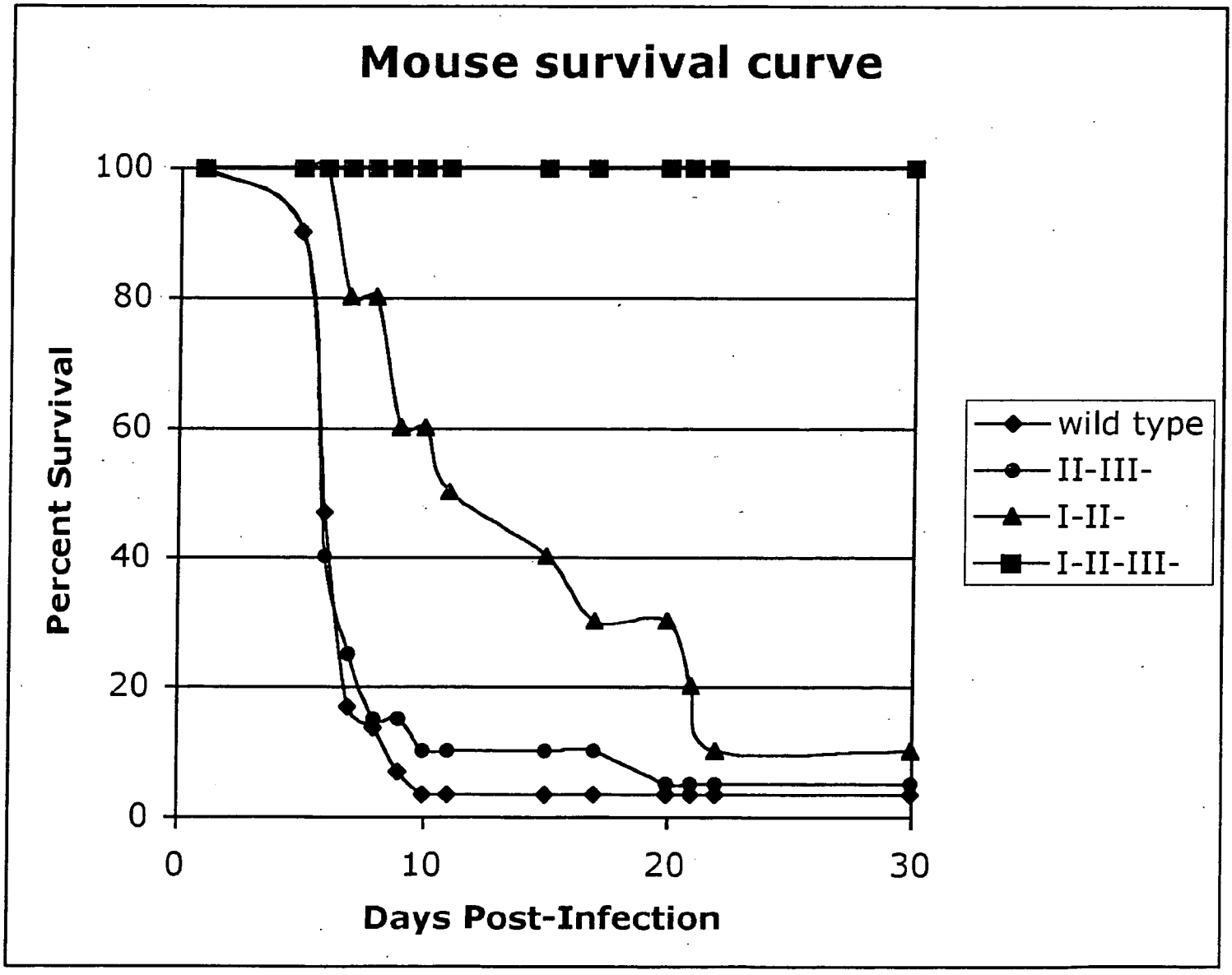
Table 2. Aerobic H_2 Oxidation Activity by *S. typhimurium* Strains

Strain	Nmoles/min/10^9 cells
wild type	13.3 ± 1.1
H_2ase I mutant	10.6 ± 0.6
H_2ase II mutant	5.2 ± 0.8
H_2ase III mutant	8.1 ± 0.7
H_2ase I^- II^-	2.0 ± 0.3
H_2ase I^- III^-	3.1 ± 0.4
H_2ase II^- III^-	0.8 ± 0.2
Triple H_2ase mutant	<0.20

The results are the mean ± std. deviation for 7 or 8 independent replicates for each strain harvested from blood agar plates; the plates had been incubated for cell growth in sealed containers in an atmosphere of 10% H_2, 5% CO_2, and balance N_2. The gas atmosphere conditions were not strictly anaerobic but were less than 0.2% O_2 partial pressure. See supplementary methods.

Figure 1. **Virulence of *S. typhimurium* strains on mice.**

The data shown is from a total of 30 mice each for the wild type and the triple mutant strain, based on the combined data from two separate experiments (see supplementary methods for strain constructions and animal virulence protocols). The inoculant was 1 x 10^6 cells introduced orally. For JSG319 (group $I^-II^-III^-$), 20 mice were used, and 10 mice for JSG315 (group I^-II^-). Data for another double mutant strain (JSG317, group I^-III^-) is not shown but was similar to that of strain JSG315 results. All mice that survived to day 30 were also still alive at day 40.


Mouse survival curve
Percent Survival
100
80
60
40
20
0
0
10
20
30
Days Post-Infection
wild type
II-III-
I-II-
I-II-III-

Abstract

Molecular hydrogen is produced in the large intestine of animals due to the fermentation reactions of sugar catabolism. The gastric pathogen *Helicobacter pylori* and the liver pathogen *Helicobacter hepaticus* have the capacity to use molecular hydrogen as a respiratory substrate. The amount of the gas within tissues colonized by these pathogens is ample, and use of H_2 significantly increases the stomach colonization ability of *H. pylori*.

Key Words: hydrogen

Helicobacter

H_2 respiration

Pathogenic bacteria

Abbreviations: cyt: cytochrome

Availability and Use of Molecular Hydrogen as an Energy Substrate for *Helicobacter species*

Robert J. Maier[1]*

[1]Department of Microbiology, University of Georgia, Athens, GA 30602, USA.
e-mail: rmaier@arches.uga.edu
fax: 706-542-2674

Hydrogen oxidation

Prokaryotes have used hydrogen gas as a high energy reducing substrate for over a billion years (1, 2). Upon binding and then "splitting" of the gas by a membrane-associated nickel-containing hydrogenase enzyme, the energy contained in the low potential electrons is conserved by a combination of transmembrane potential and proton gradient coupling mechanisms (i.e. chemiosmostic coupling). This process is facilitated by a series of quinones and (heme-containing) electron carriers, some of which are specific to the H_2 oxidizing electron transport chains (3).

The structural and mechanistic components for using H_2 as a substrate are highly similar among the diverse array of prokaryotes that can carry out this process (3,4), and coupling the respiration to O_2 as the terminal electron acceptor is the most energetically favorable for maximizing energy conservation. In addition to the structural components needed for splitting H_2 (5) and initially shuttling the electrons into membrane bound components (including for example the NiFe hydrogenase, and a heme b protein and/or a quinone binding protein), a startlingly high number of conserved "accessory protein components" are needed for maturation of hydrogenases (3,6). These play a variety of enzymatic and metal mobilizing roles that are (in most cases) unique to the maturation of hydrogenase enzymes (6,7). Therefore, the cells total expenditure for maintaining the ability to oxidize H_2 is substantial. In addition, the uptake hydrogenases are regulated in response to environmental signals (6,8), and the cells may require nickel or iron transport systems to acquire sufficient metals to incorporate into the enzyme (3,6). All of the previous reviews in this field (bacterial H_2 metabolism) view H_2 use/oxidation from the perspective of its occurrence in soil or water-borne organisms in nature. The recent

observations that some pathogenic bacteria use molecular hydrogen within animal hosts now expands the subject of respiratory hydrogen activation and oxidation to some infectious diseases, the subject herein.

Production of hydrogen within animals

When ingested carbohydrates are incompletely absorbed by the small intestine within animals, they reach the colon where they are fermented by intestinal bacteria (9,10). Many different bacteria (primarily anaerobes) present in human feces or in the colon can carry out these fermentations. The latter are known as the colonic flora. Volatile short chain fatty acids are produced from the fermentations (10); these are primarily acetate, butyrate, and propionate and are used by the host (11). Along with these fatty acids the gases H_2 and CO_2 are produced. These gases are not utilized by the host, but are primarily either lost in feces or flatus, or assimilated by methane-producing bacteria. The net hydrogen production in human fecal material is dependent on the amount of methanogenesis (consumes H_2) and the fecal stirring (affects the H_2 tension). The methane-producers compete with other bacteria (such as sulfate reducers) for available H_2 in the human colon, but it seems the methanogens predominate. Still a significant portion of the H_2 produced by the colonic flora is absorbed into the bloodstream and can be detected on the breath (12, 13). Low breath hydrogen levels are almost always associated with rapid methanogenesis.

An estimated 14% (12) or 20%(10) of the colonic H_2 is thought to be carried through the human bloodstream and released into the lungs. The amount of hydrogen detected on the breath of humans is undoubtedly related to the digestibility of the complex

carbohydrates that are consumed. For example, H_2 production on the breath of healthy adults was considerably greater when barley rich in ß-glucans was consumed compared to the control barley lacking the viscous polysaccharide (13). Also, patients with abnormally high levels of breath hydrogen were found to be taking drugs that inhibited methanogenic bacteria (14) of the colon (and therefore one of the sources of H_2 utilization). Breath H_2 analysis has even been used to determine the overall digestibility (by humans) of plant cell polysaccharides and fiber, and to determine the small bowel transit times for carbohydrate absorption (15).

Helicobacter species and hydrogenase

H. pylori is a pathogen which solely colonizes the mucosal surfaces of the human stomach, where it gives rise to gastritis, peptic ulcers and is correlated with the development of certain types of gastric cancer (16). It is a prevalent, but highly treatable infection; the most severe pathologies associated with infection are correlated with both the persistent nature of the bacterium and the highly inflammatory response of the host (17, 18). *Helicobacter hepaticus* is an enterohepatic type of *Helicobacter* that is receiving research attention due to its association with liver disease (including even liver tumors) in mice (19). The recent findings of an association of hepatic *Helicobacters* with diseased liver tissue in primates (20), and the correlations of *Helicobacter*-specific DNA with human patients having primary liver carcinomas has sparked more interest in hepatic *Helicobacter*.

Initially we determined that *H. pylori* grown in the lab with H_2 expressed a membrane bound "uptake-type" hydrogenase (21). Reduction of the membrane fraction (the

fraction that contained the bulk of the hydrogenase activity) by H_2 resulted in the membrane-associated cytochromes acquiring a reduced (ferrous) state, indicating an energy-conserving respiratory chain is operating with the electrons generated from H_2 (21). Similarly, the characterizations of the *Helicobacter hepaticus* hydrogenase (22) indicated it is coupled to H_2/O_2 respiration as well, but with a significantly lower (whole cell) activity than for *H. pylori*. Both of the *Helicobacter* spp. hydrogenases are able to couple H_2 oxidation with reduction of a variety of positive redox potential acceptors, like other uptake type hydrogenases. No H_2 evolution activity could be detected, even when low redox potential reduced dyes were supplied to the cell membranes. Also, like other characterized respiratory hydrogenases, both of the *Helicobacter* enzymes underwent reductive activation, in which highest enzyme activities are observed when the enzyme is reduced (21, 22). Some properties and comparisons of the hydrogenases of *H. pylori* and *H. hepaticus*, including affinities for H_2 and activities with O_2 as the terminal acceptor are shown in table 1. Some properties of the *H.hepaticus* hydrogenase are listed as unknown in the table, but are important aspects to be determined in the future.

Hydrogen availability and use in tissues colonized by *Helicobacter*

The levels of hydrogen gas within the intestinal tracts (primarily the hindgut) of terrestrial arthropods have been relatively well studied in order to understand microbial communities associated with digestion (23). However, H_2 levels in tissues of vertebrate animal hosts had not been assessed until we began to ascertain the importance of the substrate to *Helicobacter pylori* infection. To study the possible importance of H_2 use within animal tissues, we assayed H_2 levels in live mice. Both the mucous lining of the

stomach (24) as well as the lobes of the liver (22) contained ample amounts of molecular H_2, certainly higher levels than we had anticipated. The average hydrogen content of the mucus layer of the mouse stomach was determined to be 43 μM (range of 17 to 93 μM), averaging over 20 -fold that of the whole cell K_M for hydrogen. Similarly, the H_2 levels in liver tissue were 20-fold higher than the *H. hepaticus* Km for H_2. These measurements in the animal were taken by use of H_2 microelectrode inserted into live mice, and it was proposed that these H_2 levels were achieved by a combination of cross-epithelial diffusion of the gas from the bowel, and from H_2 carried in the bloodstream (24). By combining the tissue H_2 measurements with studies on the binding affinity of the bacteria for H_2 (conducted under limiting substrate conditions) it was concluded that *Helicobacter* hydrogenase is saturated with H_2 in the host tissues.

A mutant *H. pylori* strain unable to oxidize hydrogen is severely impaired in its ability to colonize mice (24). A total of 9 mice out of 38 inoculated with the mutant strain contained any detectable *Helicobacter* in their stomach, whereas the parent strain colonized every inoculated mouse. Moreover, for those scored as colonization positive by the mutant the colonization numbers (colony forming units per g of stomach) were markedly less for the mutant than the parent strain. These results indicate that H_2 is a major, although not the sole, utilizable energy substrate used by *H. pylori*. The results are convincing that H_2 use by *H. pylori* is an important maintenance factor for its survival in the host. The importance of H_2 use to *H. pylori* colonization capability represents a new aspect toward our understanding of host-pathogen relationships. Molecular hydrogen use was not formerly recognized as a factor in understanding how a human pathogen grows within an animal host. *Helicobacter hepaticus* mutants have not been

obtained, so studies on the affects of H_2 use on *H. hepaticus* colonization of (mouse) liver have not been possible.

It is not yet known whether H_2 oxidation by *Helicobacter* provides ATP, reductant, or a transmembrane potential that is used for other work. There is no evidence from the genome sequence information that *H. pylori* is an chemoautotroph (uses oxidizable energy sources to fix/assimilate carbon dioxide), but many H_2 oxidizers do use H_2 for this purpose. Therefore, carbon sources must be obtained from the assimilation of organic molecules, and these could include sugars, small peptides or amino acids. It is possible that the energy from H_2 oxidation is used specifically to transport organic molecules. There is precedent for H_2 oxidation/respiration playing a major role in driving bacterial sugar transport (25).

Regulation of Hydrogenase

One characteristic of the bacteria capable of expressing energy-conserving uptake hydrogenases is their ability to sense and then respond (by altering hydrogenase gene expression) to exogenously supplied hydrogen (6, 8). Hydrogenase activity in *H. pylori* was constitutive under all conditions tested (in rich media), but in a chemically defined media, the activity increased 4-fold when the cells were supplemented with 10% H_2 (24). Promoter fusions with a reporter gene were used to address this regulation at the transcriptional level; a 6-fold increase in transcriptional response was observed by the H_2 exposure. The enzyme expression response to molecular hydrogen availability is in line with the conclusion that hydrogenase of *Helicobacter* functions in respiratory hydrogen oxidation.

Microaerobic Hydrogen Oxidation and Respiration

Coupling of H_2 oxidation all the way to O_2 would be the most energetically favorable for harvest of the electrons, but some bacteria are unable to do so. H_2 oxidation by the *Helicobacter* species (*pylori* and *hepaticus*) was observed to be coupled to O_2 reduction (21, 22) and anaerobic growth of *Helicobacter pylori* has never been demonstrated. An important concern is whether the terminal oxidases of these bacteria are sufficient to operate in the low free O_2 environment of the gastric mucus or the liver tissue. The ability of the H_2-mediated respiratory chain to generate ATP will depend on the overall electron flux through the chain, thus it could depend on the ability of the terminal oxidase to bind and reduce O_2. A terminal oxidase of the *cbb*$_3$ type has been studied from *H. pylori* and it would be expected to oxidize cytochrome c and to function in low O_2 levels (such as in blood or tissue). A low Km for the *H. pylori cbb*$_3$ oxidase was reported and it is expected that this oxidase could function in nM levels of free O_2 (26).

From absorption spectral studies on a clinical isolate (from a duodenal ulcer patient) evidence for a cyt b/d terminal oxidase in *H. pylori* membranes was obtained (21). However, the complete genome sequence of two strains (strain 26695 and J99) does not show the existence of a b/d type oxidase (27). It was thus concluded by authors of a review (28) that the clinical isolate work is at variance with many other authors regarding our knowledge of *H. pylori* respiration. However, it must be remembered that strain variations in phenotype and genotype are common for *H. pylori*, and that *Campylobacter jejuni* (closely related to *H. pylori*) does contain the cyt b/d type oxidase. Also, genome sequencing of *H. hepaticus* shows that the liver-colonizing *Helicobacter* does contain the

cyt b/d type oxidase (Suerbaum: Pers communication). Like the *cbb*$_3$ type of cyt c oxidase, the cyt b/d type quinol oxidases are also well suited to function in microaerobic environments.

Outlook and Perspective

A wide range of characteristics are attributed to infectious bacteria that can be called virulence determinants to successfully combat host protection mechanisms. Many of these are secreted toxins or inflammation-eliciting determinants (29), or in specialized cases, enzymes (like urease) that modify the colonization environment to make it permissive for the pathogen. However, the primary sources of energy used by infectious bacteria to sustain their growth, once they are established in an animal host, remain largely unknown (29). Molecular H_2 is an energy substrate not used by the host, so competition for this high-energy substrate in the gastric environment is not a factor. *Helicobacter* has retained an ancient (2) energy-conserving pathway to support its energy needs within the host.

Hydrogen use is expected to play an important role in setting up the stable infections required for the most serious of the diseases associated with *H. pylori* infection. Colonization and persistence occurs within the complex and viscous mixture of glycoproteins known as mucin. According to physiological studies as well as from the complete genome sequence information, *H. pylori* appears to be limited in its use of oxidizable carbon substrates (27), and the primary environment for *H. pylori* is also expected to be nutrient poor in regards to energy sources available for growth and

maintenance. Molecular hydrogen use would thus seem to circumvent some of the colonization barriers faced by *H. pylori*.

Due to the above considerations, it would be expected that (increased) *H. pylori* infection might be correlated with (H_2 producing) diet regimes. The proportion of exhaled gas as H_2 can vary considerably among individuals so it may be possible someday to correlate *H. pylori* infection with inherent host H_2-production characteristics. The prevalence of *H. pylori* infection is thought to depend in part on environmental factors, including diet, age of the individual, or the genetic make-up of the individual or population. An obvious question is: could diet alterations (and thus H_2 production) be a treatment procedure to rid an individual of (H_2 utilizing) pathogens? Considering the high affinities of H_2 oxidizing hydrogenases for their substrate, H_2 levels would have to be reduced within the individual to sub-μM levels, and our data (for mice) indicates levels in tissues are in the 40—60 μM range. Therefore, the microbial fermentations by the colonic flora would have to be dramatically reduced by diet in order to starve a pathogen of (hydrogen) energy. Reducing or eliminating such flora is unwise as such bacteria of course have beneficial attributes for animal nutrition and digestion. A better approach to reduce H_2 consumption by pathogens may be to design inhibitors of the H_2 utilizing hydrogenases. These enzymes are oftentimes periplasmic in location and the enzyme contains a highly unique active center (5) containing Ni and Fe with attached CN and CO ligands (30). Therefore we should expect few host affects by use of NiFe active site specific inhibitors. Another approach could be to starve the pathogen of nickel, an essential element for the uptake-type hydrogenases (and for another virulence component, urease).

Other Hydrogen-utilizing Pathogens

That hydrogen present in animals (as a consequence of normal colonic flora metabolism) is an energy yielding substrate for maintenance of a pathogenic bacterium may be extended to a number of (mostly enteric) pathogens. Based on publicly available complete genome sequence annotations (such as the Institute for Genomic Research, the Sanger Institute, or the National Center for Biotechnology Information) a number of human pathogens have genes encoding all the components required for gleaning energy from H_2 respiration. This would include the structural genes for a membrane bound hydrogenase and for shuttling of those electrons to quinone-binding or heme b binding proteins, as well as the accessory proteins for the NiFe hydrogenase enzymes' maturation. These bacteria include *Salmonella enterica* serovars *Typhi* and *Typhimurium*, *E. coli* 0157, *Shigella* (*flexneri* and *sonnei*) and *Campylobacter jejuni.* For some of the enterics, H_2 oxidation via hydrogenases has been measured (albeit anaerobically). Most of these pathogens live in the gastrointestinal tract, or within organs that would have ample blood supply (that presumably contains H_2). Most of the above bacteria also contain the complete respiratory electron transport chain (normally used in common by H_2 and other low potential electrons donors), including one or more O_2-binding terminal oxidases. If these bacteria are able to couple H_2 oxidation to O_2 uptake, it would be expected they could all gain considerable maintenance advantage in colonizing the host. Also, many non-pylori *Helicobacters* (20) live in the gastrointestinal tracts of animals, including

humans. The extent to which they use molecular hydrogen is not known, but they exist (colonize the host) closer to the original source of H_2 (the fermentative bacteria) than either *H. pylori* or *H. hepaticus*. Further studies are needed to ascertain the usefulness of H_2 as an energy source for other pathogenic bacteria and other *Helicobacter* species.

Acknowledgments

The author's recent work was supported by the Georgia Research Foundation, and the National Institute of Health. A major contributor to the original concepts and results discussed herein is Dr. Jonathan Olson of North Carolina State University.

References

[1] J. Alper, Putting an Exotic Enzyme together, ASM News, 69 (2003) 170-171.

[2] M.W.W. Adams, (Ed) Enzymes and proteins from hyperthermophilic microorganisms; Advances in protein chemistry, vol. 48, Academic Press, San Diego, CA, 1996.

[3] P.M. Vignais, B. Billoud,, J. Meyer, Classification and phylogeny of hydrogenases, FEMS Microbiol. Rev. 25 (2001) 455-501.

[4] L. Casalot, M. Rousset, Maturation of the [NiFe] hydrogenases, Trends in Microbiol. 9 (2001) 228-237.

[5] S.P.J. Albracht, in : L.G. Ljungdahl, M.W. Adams, L.L.Barton, J.G. Ferry, M.K. Johnson (Eds), Biochemistry and physiology of anaerobic bacteria, Springer-Verlag, New York, 2003, pp. 20-34.

[6] R.J. Maier, J. Olson, N. Mehta, in: L.G. Ljungdahl, M.W. Adams, L.L.Barton, J.G. Ferry, M.K. Johnson (Eds), Biochemistry and physiology of anaerobic bacteria, Springer-Verlag, New York, 2003, pp. 67-84.

[7] M. Hube, M. Blokesch, A. Bock, Network of hydrogenase maturation in *Escherichia coli*: role of accessory proteins HypA and HybF, J. Bacteriol. 184 (2002) 3879-3885.

[8] L.K. Black, C. Fu, R.J. Maier, Sequences and characterization of hupU and hupV genes of *Bradyrhizobium japonicum* encoding a posssible nickel-sensing complex involved in hydrogenase expression, J. Bacteriol. 176 (1994) 7102-7106.

[9] J.H. Cummings, Fermentation in the human large intestine:evidence and implication for health, Lancet 1 (1983) 1206:1209.

[10] M.J. Wolin, T.L. Miller, Acetogenesis from CO_2 in the human colonic ecosystem, in:H.L. Drake (Ed.), Acetogenesis, Chapman & Hall, New York, N.Y., 1994, pp. 365-385.

[11] T.L. Miller, M.J. Wolin, Pathways of acetate, propionate, and butyrate formation by the human fecal microbial flora, AEM, 62 (1996) 1589-1592.

[12] J.H. Bond, Jr., M.D. Levitt, R. Prentiss, Investigation of small bowel transit time in man utilizing pulmonary hydrogen (H_2) measurements, J. Lab. Clin. Med., 85 (1975) 546-555.

[13] C.H. Lifschitz, M.A Grusak, N.F. Butte, Carbohydrate digestion in humans from a ß-glucan-enriched barley is reduced, Human Nutrition and Metabolism, J. Nutr. (2002) 2593-2596.

[14] T.H. Florin, Alkyl halides, super hydrogen production and the pathogenesis of pneumatosis cystoides coli, Gut 41 (1997) 778-784.

[15] R.N. Tharanathan, Food-derived carbohydrates—structural complexity and functional diversity, Crit. Rev. Biotechnol. 22 (2002) 65-84.

[16] M.J. Blaser, *Helicobacter pylori* and gastric diseases, BMJ, 316 (1998) 1507-1510.

[17] D.J. McGee, H.L.T. Mobley, Mechanisms of *Helicobacter pylori* infection: Bacterial factors, Curr. Top. Micorbiol. Immunol. 241 (1999) 155-180.

[18] E. Touati, V. Michel, J.M. Thiberge, N. Wuscher, M. Huerre, A. Labigne, Chronic *Helicobacter pylori* infections induce gastric mutations in mice, Gastroenterology 124 (2003) 1408-1419.

[19] J.V. Solnick, D.B. Schauer, Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enteropepatic diseases, Clin. Microbiol. Rev. 14 (2001) 59-97.

[20] J.G. Fox, The non-H pylori *helicobacters*: their expanding role in gastrointestinal and systemic diseases, Gut 50 (2002) 273-283.

[21] R.J. Maier, C. Fu, J. Gilbert, F. Moshiri, J. Olson, A.G. Plaut, Hydrogen uptake hydroganse in *Helicobacter pylori*, FEMS Microbiol Lett. 141 (1996) 71-77.

[22] R.J. Maier, J. Olson, A. Olczak, Hydrogen-oxidizing capabilities of *Helicobacter hepaticus* and in vivo availability of the substrate, J. Bacteriol. 185 (2003) 2680-2682.

[23] T. Lemke, T. van Alen, J.H.P. Hackstein, A. Brune, Cross-epithelial hydrogen transfer from the midgut compartment drives methanogenesis in the hindgut of cockroaches, AEM, 67 (2001) 4657-4661.

[24] J.W. Olson, R.J. Maier, Molecular hydrogen as an energy source for *Helicobacter pylori*, Science, 298 (2002) 1788-1790.

[25] R.J. Maier, J. Prosser, Hydrogen-mediated mannose uptake in *Azotobacter vinelandii*, J. Bacteriol. 170 (1988) 1986-1989.

[26] D.J. Kelly, N.J. Hughes, R.K. Poole, in H.L.T. Mobley, G.L. Mendz, S.L. Hazell (Eds.), *Helicobacter pylori*, physiology and genetics, ASM Press, Washington D.C., 2001, pp. 113-124.

[27] P. Doig, B.L. deJonge, R.A. Alm, E.D. Brown, M. Uria-Nickelsen, B. Noonan, S.D. Mills, P. Tummino, G. Carmel, B.C. Guild, D.T. Moir, G.F. Vovis, T.J. Trust, *Helicobacter pylori* physiology predicted from genomic comparison of two strains, Microbiol. & Mole. Biol. Rev. 63 (1999) 675-707.

[28] M.A. Smith, M. Finel, V. Korolik, G.L. Mendz, Characteristics of the aerobic respiratory chains of the microaerophiles Campylobacter jejuni and Helicobacter pylori, Arch Microbiol 174 (2000) 1-10.

[29] A. Salyers, D.D. Whitt, Bacterial Pathogenesis: A molecular approach, ASMPress, Washington D.C. 2002.

[30] S. Reissman, E. Hochleitner, H. Wang, A. Paschos, F. Lottspeich, R.S. Glass, A. Bock, Taming of a poison: biosynthesis of the NiFe-hydrogenase cyanide ligands, Science 299 (2003) 1067-1070.

All patents and other publications cited herein are expressly incorporated herein by reference.

CLAIMS

1. A bacterium having one or more hydrogenase enzymes which oxidize H_2, the bacterium comprising:

one or more mutations in the amino acid sequence of at least one of the hydrogenase enzymes, wherein the one or more mutations substantially prevents the hydrogenase enzymes from oxidizing H_2.

2. The bacterium of claim 1, wherein:

said bacterium is Salmonella typhimurium.

3. The bacterium of claim 1, wherein:

said bacterium is Salmonella typhi.

4. The bacterium of claim 1, wherein:

said bacterium is E. coli 0157.

5. The bacterium of claim 1, wherein:

said bacterium is Shigella flexneri.

6. The bacterium of claim 1, wherein:

said bacterium is Shigella sonnei.

7. The bacterium of claim 1, wherein:

said bacterium is Campylobacter jejuni.

8. A bacterium having three membrane bound hydrogenase enzymes which oxidize H_2, the bacterium comprising:

one or more mutations in the amino acid sequence of each one of the three memberane bound hydrogenase enzymes, wherein the one or more mutations substantially prevents the hydrogenase enzymes from oxidizing H_2.

9. The bacterium of claim 8, wherein:

said bacterium is Salmonella typhimurium.

10. The bacterium of claim 8, wherein:

said bacterium is Salmonella typhi.

11. The bacterium of claim 8, wherein:

said bacterium is E. coli 0157.

12. The bacterium of claim 8, wherein:

said bacterium is Shigella flexneri.

13. The bacterium of claim 8, wherein:

said bacterium is Shigella sonnei.

14. The bacterium of claim 8, wherein:

said bacterium is Campylobacter jejuni.

15. A method vaccinating a mammal, the method comprising:

inoculating the mammal with a bacterium which includes (i) three membrane bound hydrogenase enzymes which oxidize H_2 and (ii) one or more mutations in the amino acid sequence of each one of the three memberane bound hydrogenase enzymes, wherein the one or more mutations substantially prevents the hydrogenase enzymes from oxidizing H_2.

16. The bacterium of claim 15, wherein:
said bacterium is Salmonella typhimurium.

17. The bacterium of claim 15, wherein:
said bacterium is Salmonella typhi.

18. The bacterium of claim 15, wherein:
said bacterium is E. coli 0157.

19. The bacterium of claim 15, wherein:
said bacterium is Shigella flexneri.

20. The bacterium of claim 15, wherein:
said bacterium is Shigella sonnei.

21. The bacterium of claim 15, wherein:
said bacterium is Campylobacter jejuni.

22. A vaccination, comprising:

a bacterium having three membrane bound hydrogenase enzymes which oxidize H_2, the bacterium comprising:

a bacterium which includes (i) three membrane bound hydrogenase enzymes which oxidize H_2 and (ii) one or more mutations in the amino acid sequence of each one of the three memberane bound hydrogenase enzymes, wherein the one or more mutations substantially prevents the hydrogenase enzymes from oxidizing H_2.

23. A Salmonella typhimurium mutant, comprising:

deletions, 1 - (STM 3150, STM 3149, STM 3148, STM 3147), 2 - (STM 1539, STM 1538), and 3 - (STM 1786, STM 1787).

24. A vaccination, comprising:

a Salmonella typhimurium mutant having deletions, 1 - (STM 3150, STM 3149, STM 3148, STM 3147), 2 - (STM 1539, STM 1538), and 3 - (STM 1786, STM 1787).